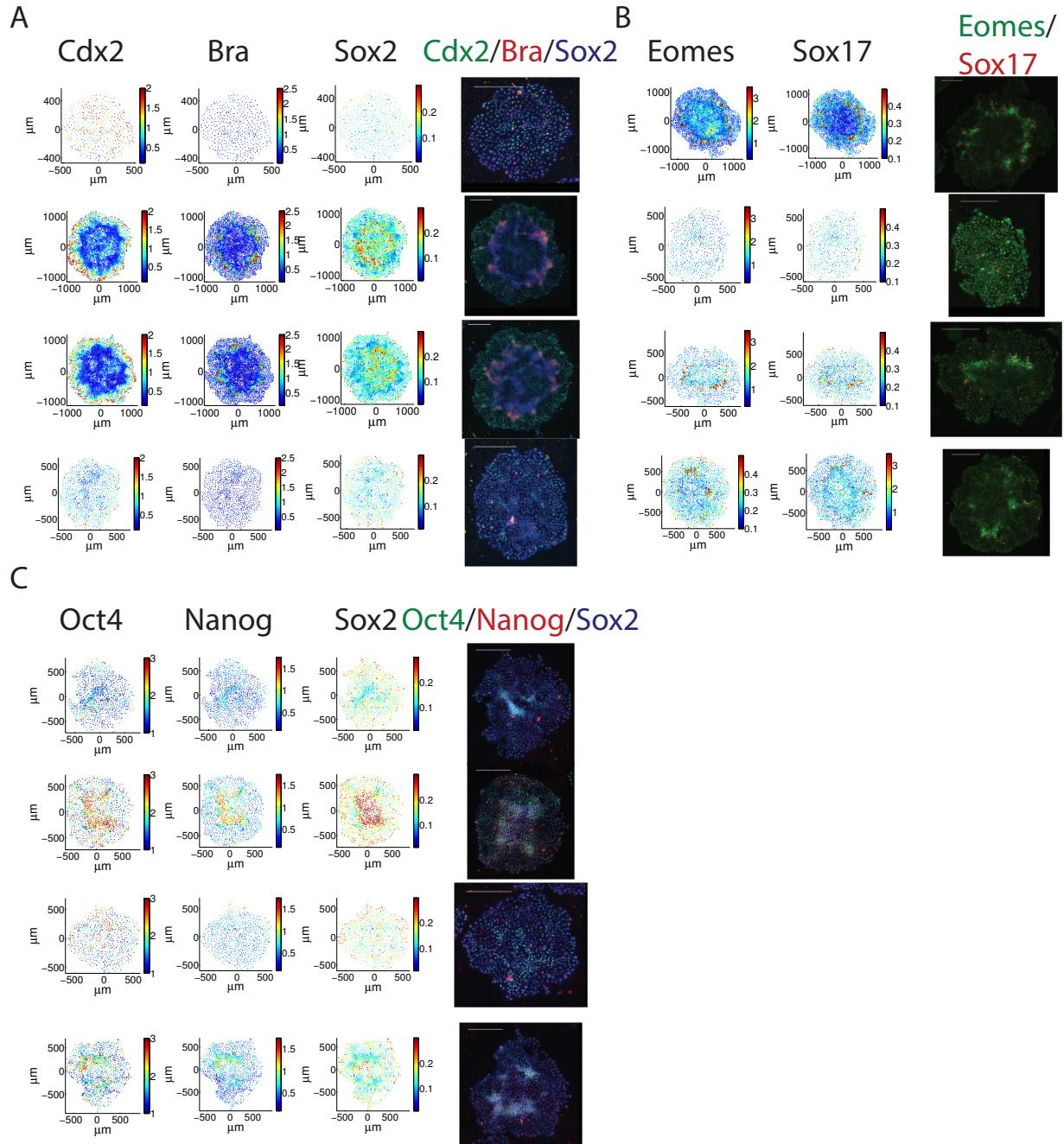
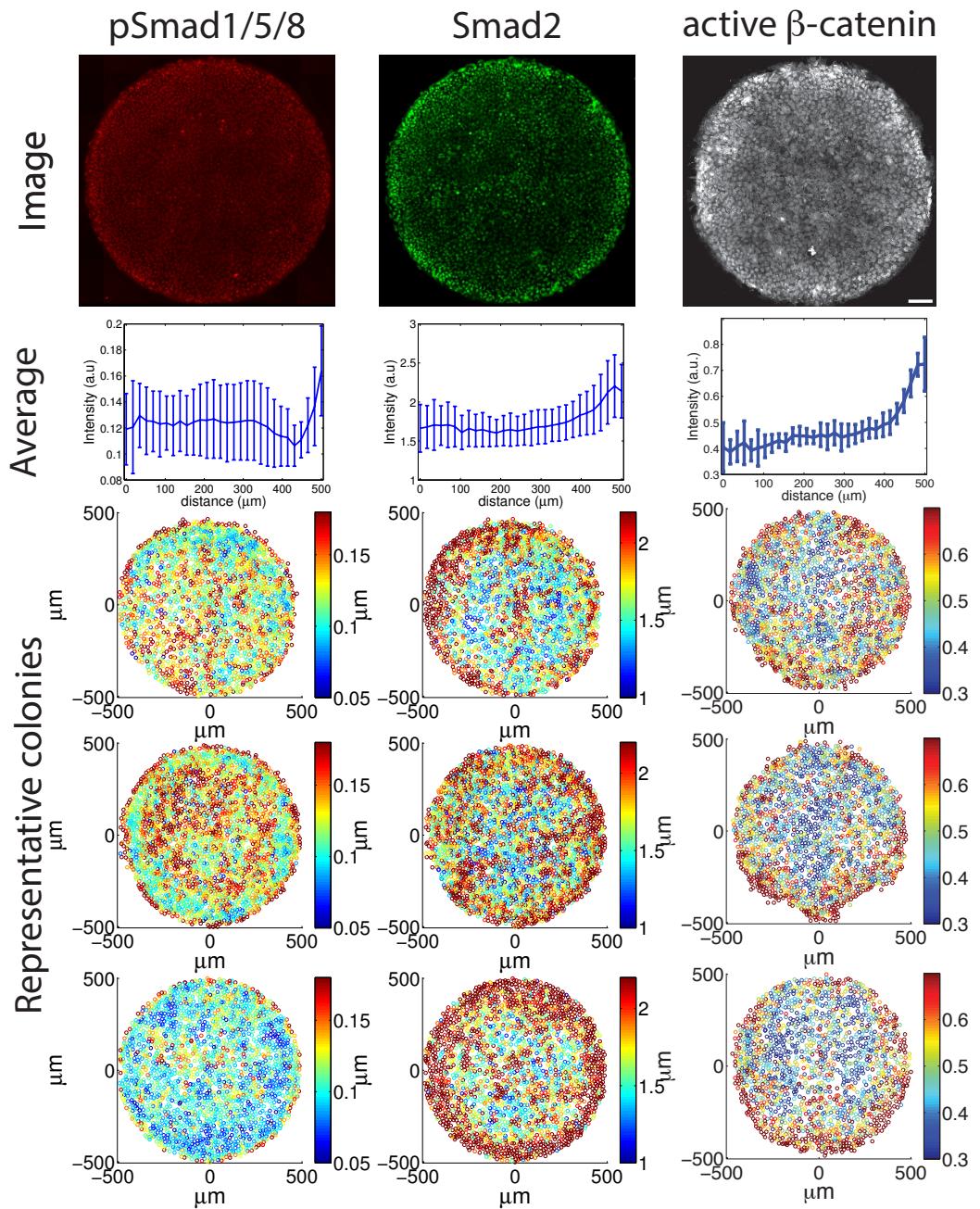


Supplementary Figure 1.



BMP4 leads to inconsistent patterns of differentiation under standard culture conditions. Cells were grown in well-separated colonies. In each panel, each row shows the quantification and merged image for a single colony. Each dot represents a single cell quantified for the indicated marker. All colonies were differentiated with 50 ng/ml BMP4 for 42 hours. All scale bars are 500μm.

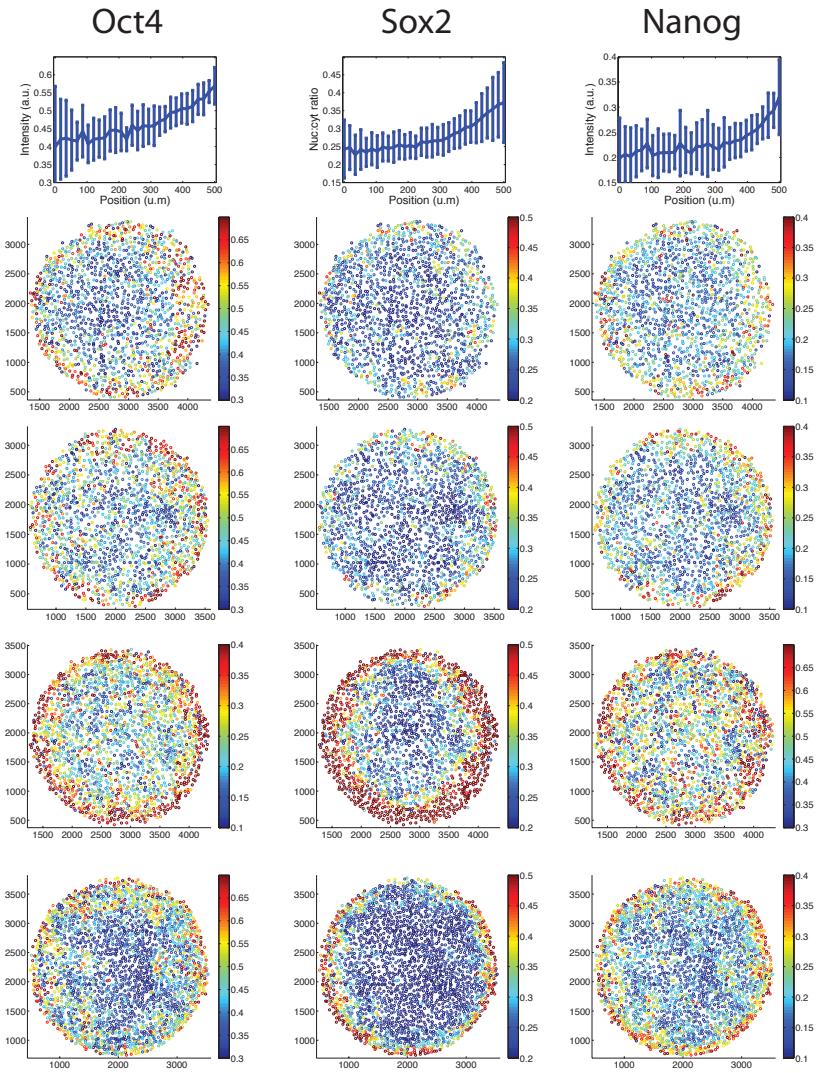
Supplementary Figure 2.



The Activin/Nodal, BMP, and Wnt signaling pathways are more active at the colony edge in the state of pluripotency. Cells were seeded on micropatterned coverslips and grown overnight before fixation and staining with antibodies for pSmad1, Smad2, or activated β -catenin. Cells were also stained with DAPI to identify individual cells. The amount of pSmad1 or activated β -catenin in each cell was normalized to the DAPI intensity while the amount of nuclear Smad2 was normalized to cytoplasmic Smad2. This normalization was shown to be a reliable assay for TGF- β activity in ref¹. The top row show images of representative colonies, while the second row shows the image quantification

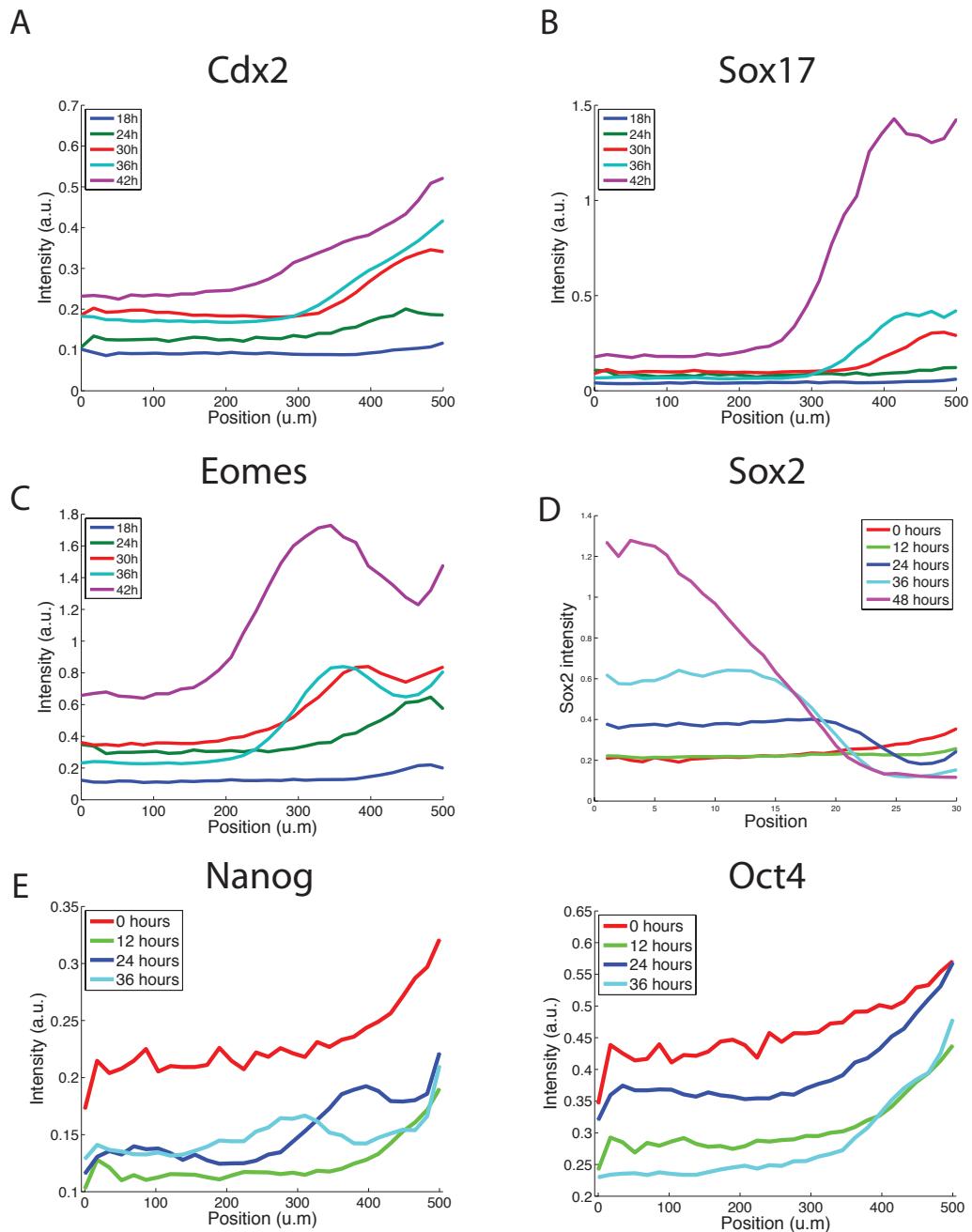
averaged over all colonies. Error bars indicate standard deviations across colonies. The bottom three rows show quantifications of representative colonies – each dot represents a cell. The position of the cells within the colony is plotted on the axes, while the color corresponds to the quantified signaling intensity for the indicated pathway. The color scale is consistent for the three colonies corresponding to each marker.

Supplementary Figure 3.



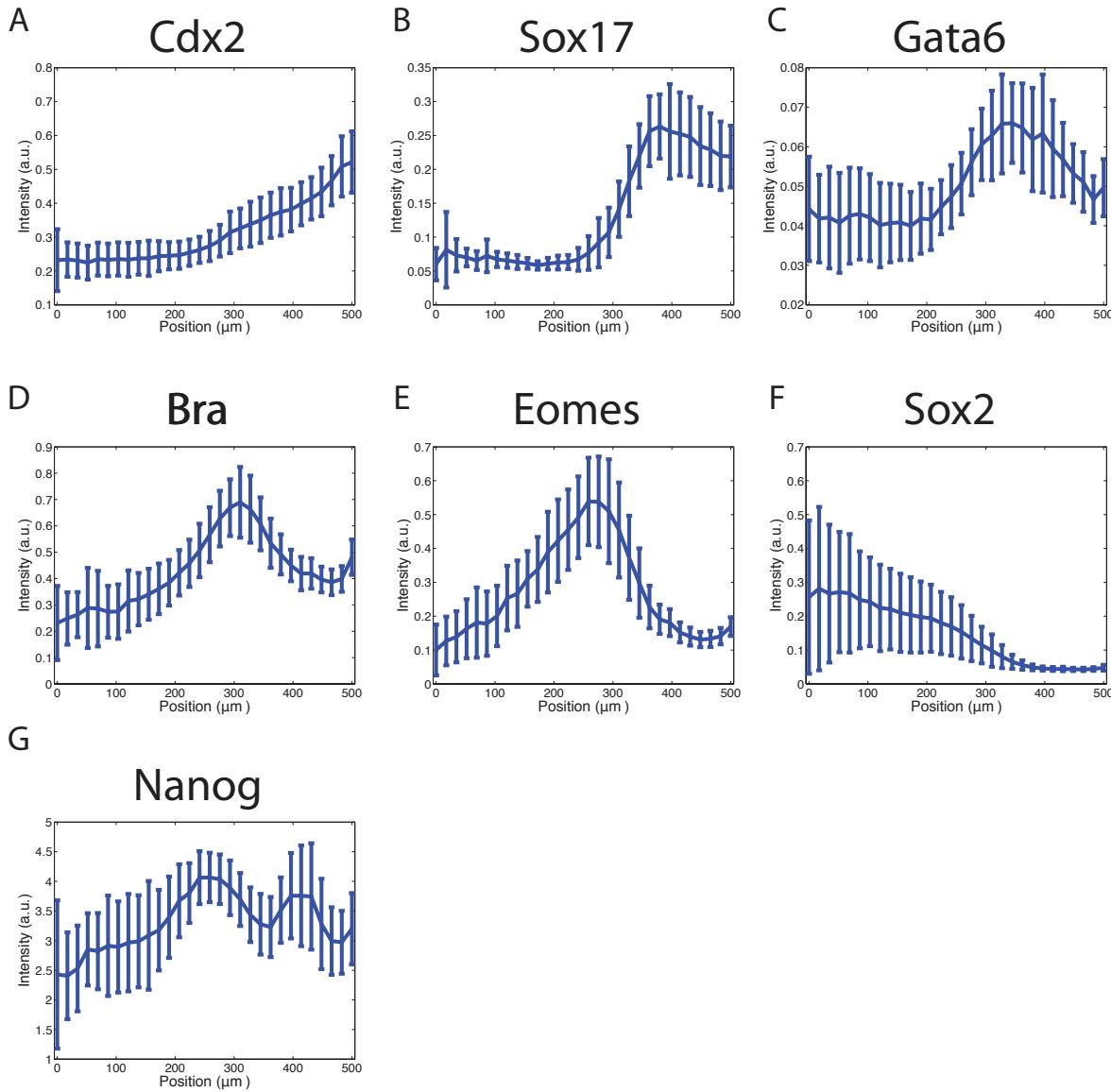
The pluripotency markers Oct4, Sox2 and Nanog are more highly expressed at the colony edge in a reproducible manner. Cell preparation and image quantification was identical to that in Supplementary Figure 2. The top row shows averages and standard deviations across all colonies while the lower rows show individual colonies quantified cell-by-cell for each marker. Each row represents a single colony quantified for all three markers as in Supplementary Figure 2.

Supplementary Figure 4.



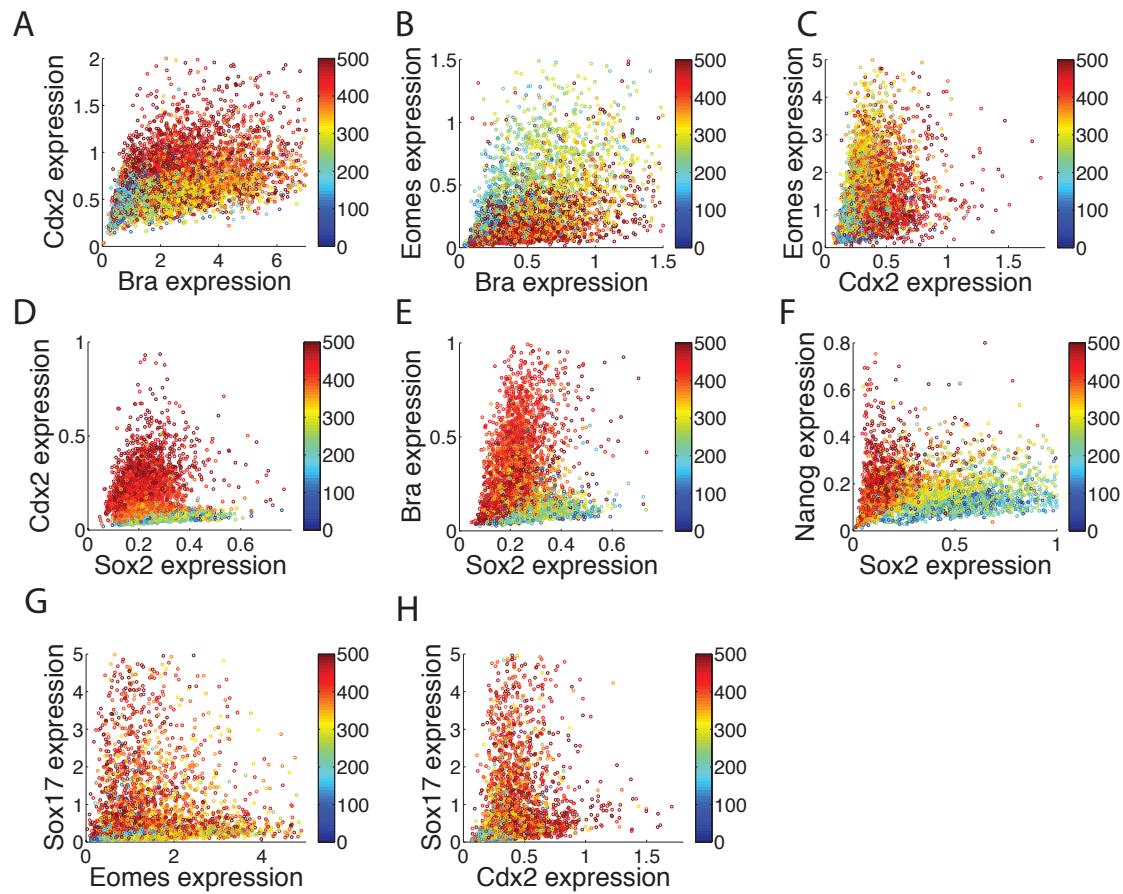
Dynamics of pluripotency and differentiation markers. Markers were quantified as in Supplementary Figure 3 at the indicated times. The results show that differentiation begins at the colony edge and mesendodermal markers then move inwards within the colony. In contrast, Sox2 expression falls initially at the colony edge and then rises at the center of the colony.

Supplementary Figure 5.



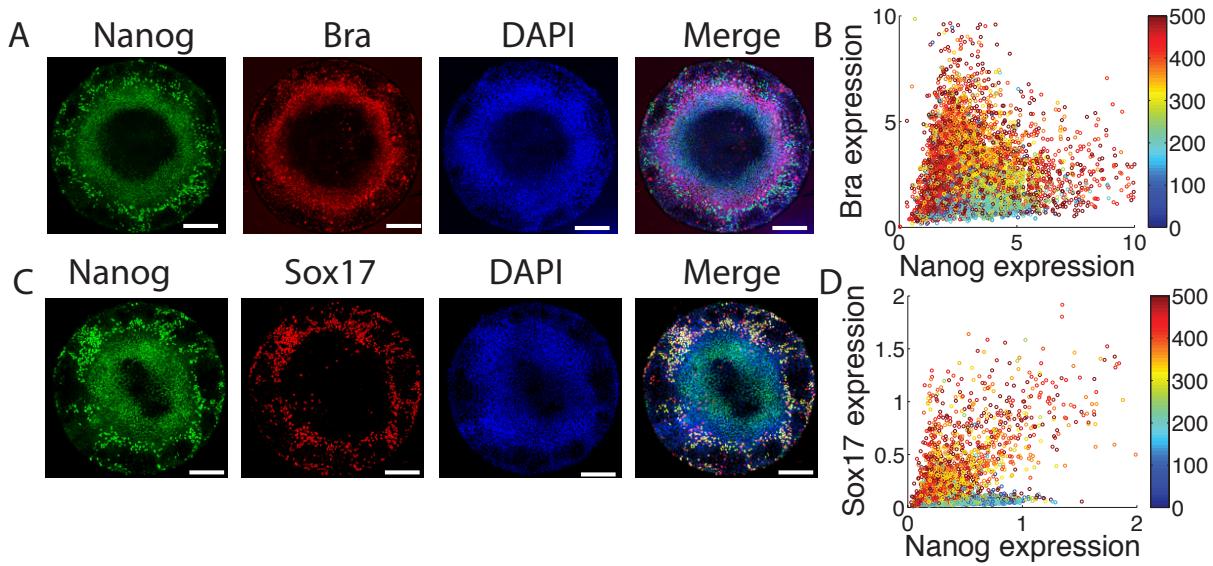
Differentiation patterns are reproducible. Cells were seeded on micropatterned coverslips grown for one day and then differentiated with BMP4 for 42 hours. Patterns of differentiation were quantified cell by cell and then each colony was averaged over the angular coordinate to produce a radial profile. Plots show the average radial profile and standard deviation across colonies. The larger error bars on Sox2 result from the displacement of the Sox2 expressing territory from the exact center of the colony in several cases. Also a small number of colonies failed to express Sox2 (2 out of 23 colonies) and in these colonies the mesodermal cells extended to the center of the colony.

Supplementary Figure 6.



Single cell coexpression analysis. Cells were differentiated with BMP4 for 42 hours and quantified for the indicated markers by immunofluorescence. Each point represents a single cell quantified for the fate markers indicated on the x- and y-axes. The color scale indicates the position of the cell in microns from the colony center. For each plot, data were pooled from all the 1000 μ m diameter colonies on a single coverslip.

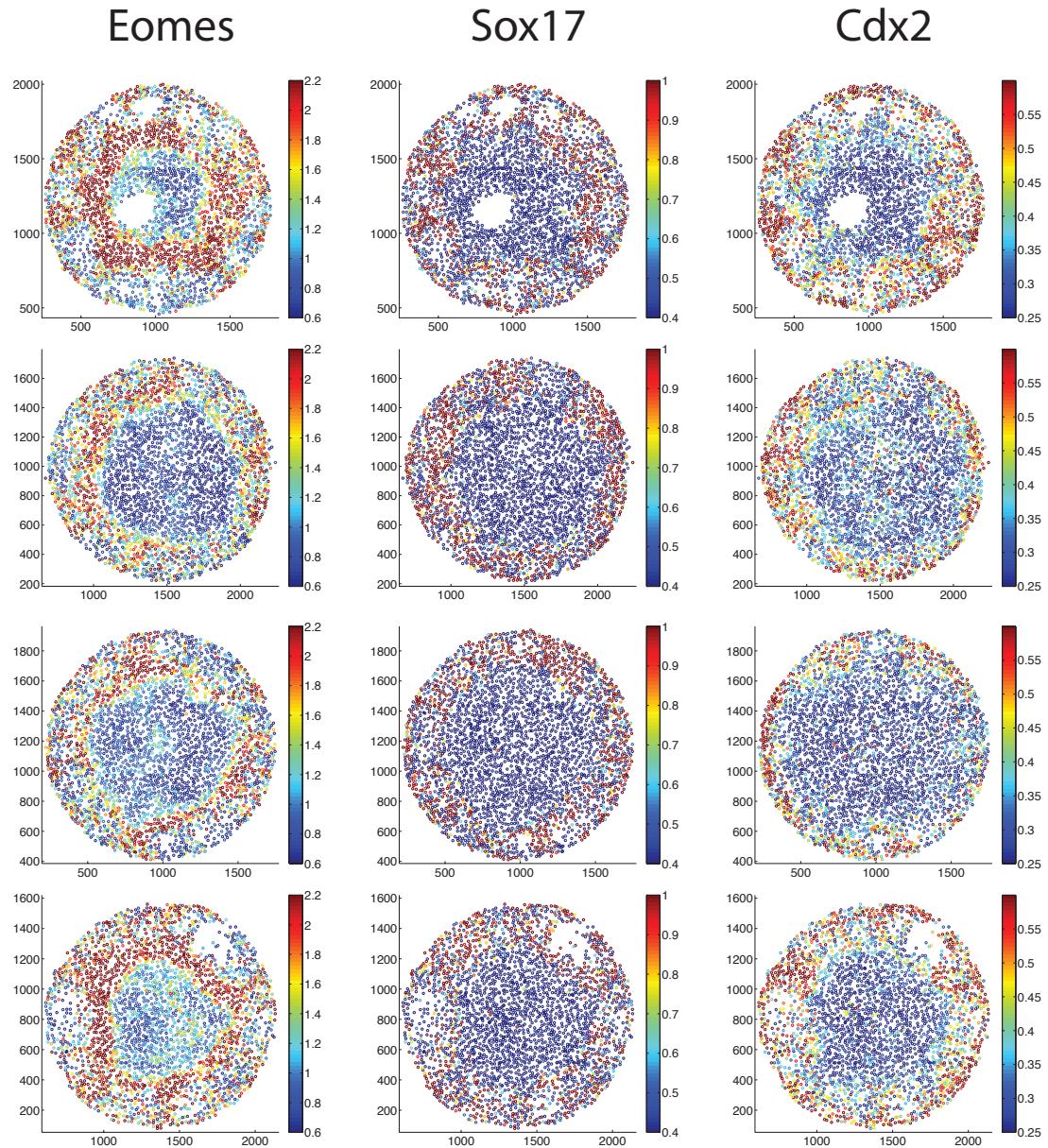
Supplementary Figure 7.



Nanog is coexpressed with Brachyury and Sox17 during mesendoderm

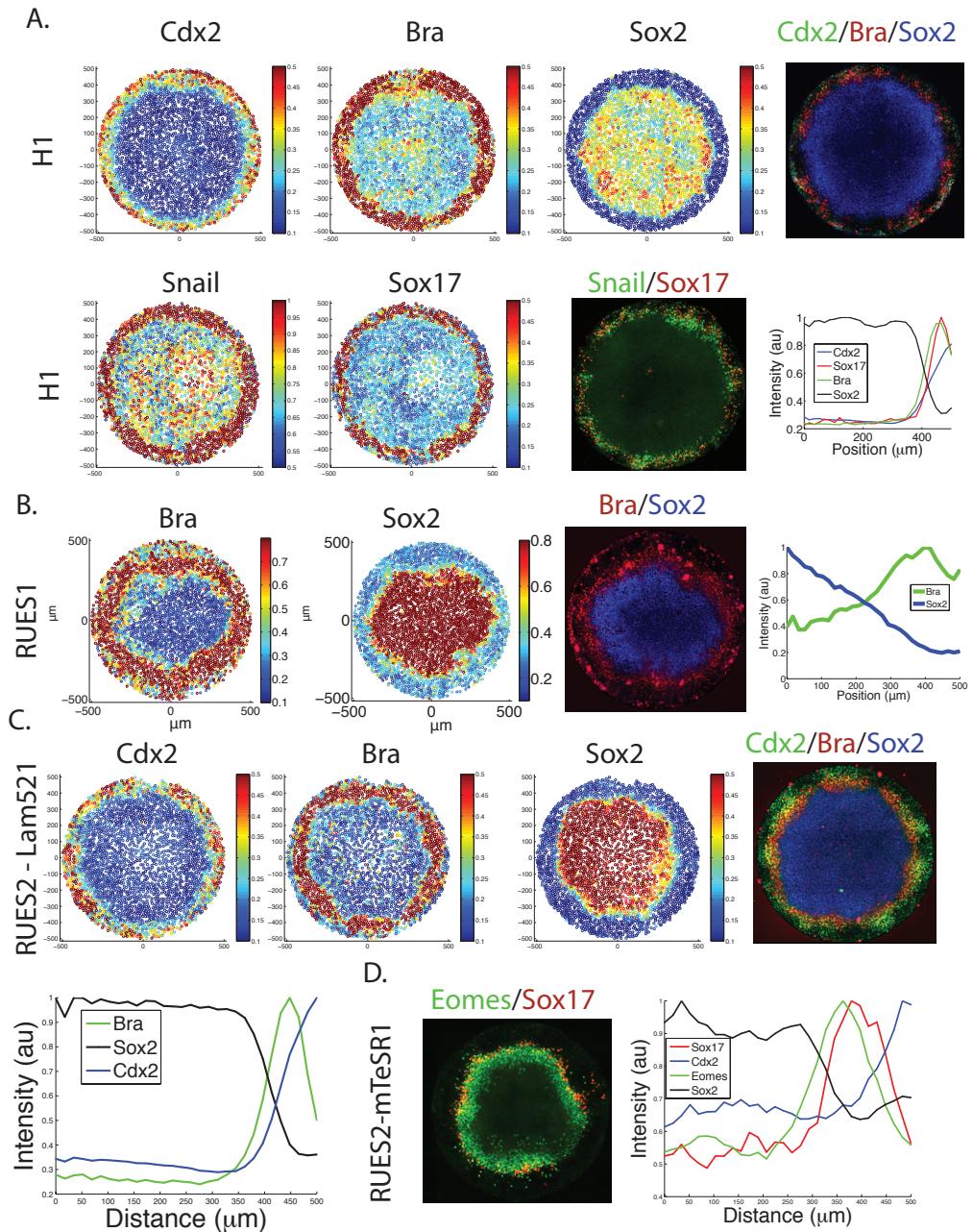
differentiation. Cells were differentiated with BMP4 for 42 hours and analyzed by immunofluorescence. **(A-B)** Nanog is expressed in a ring together with Brachyury. **(C-D)** Nanog is particularly highly expressed in cells that also express Sox17. Scale bars 200 μ m. Plots in **(B,D)** were made as in Supplementary Fig. 6.

Supplementary Figure 8.



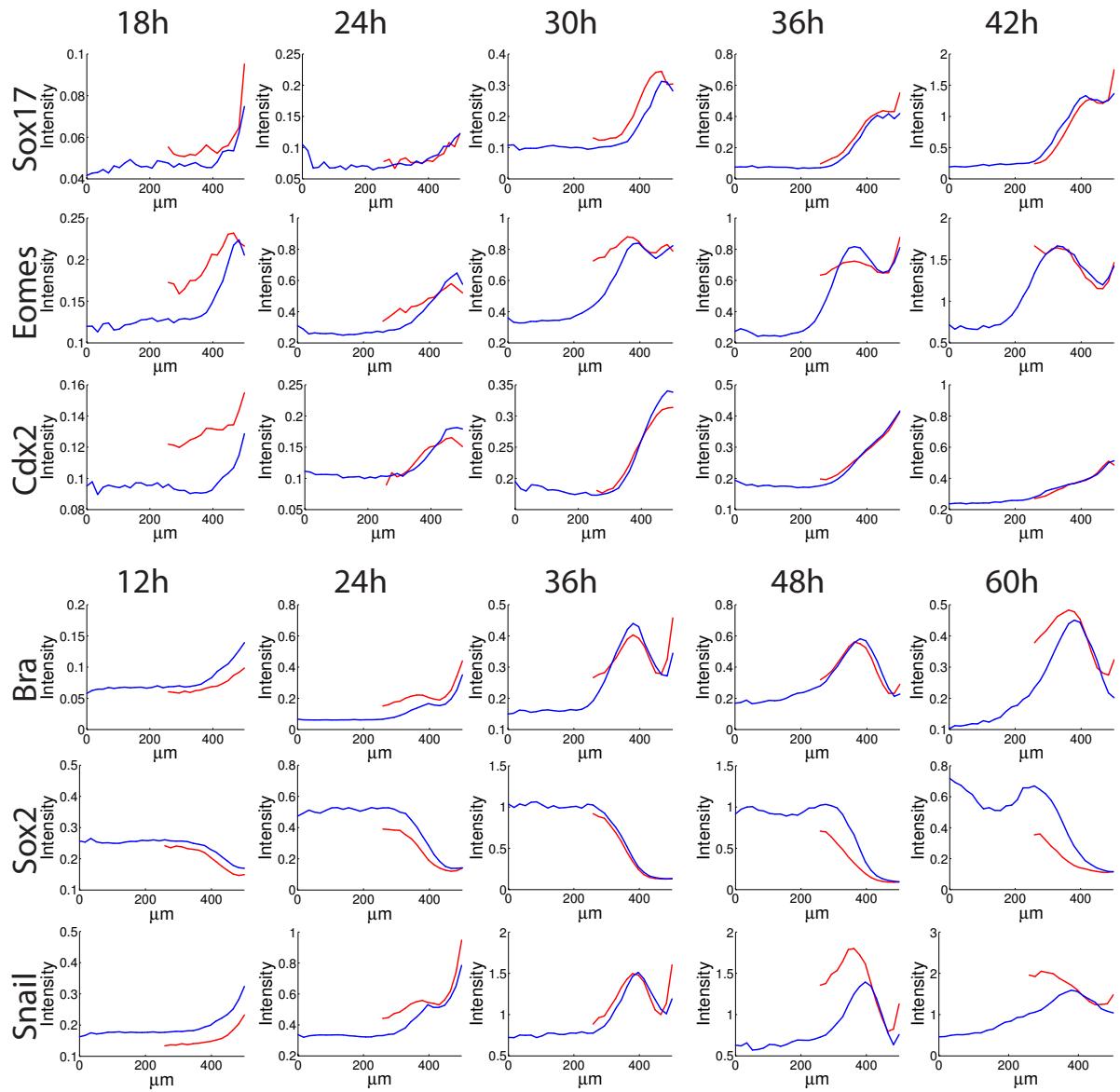
Quantification of differentiation patterns in individual 1000 μ m colonies. Each row represents a single colony triple stained for the indicated markers as in Supplementary Fig. 3. Each dot in the plot represents a cell. The holes in the first and last colony are adhesion problems presumably due to nonuniformity in substrate preparation and occur in approximately 10% of colonies. They do not perturb the averaged radial expression.

Supplementary Figure 9.



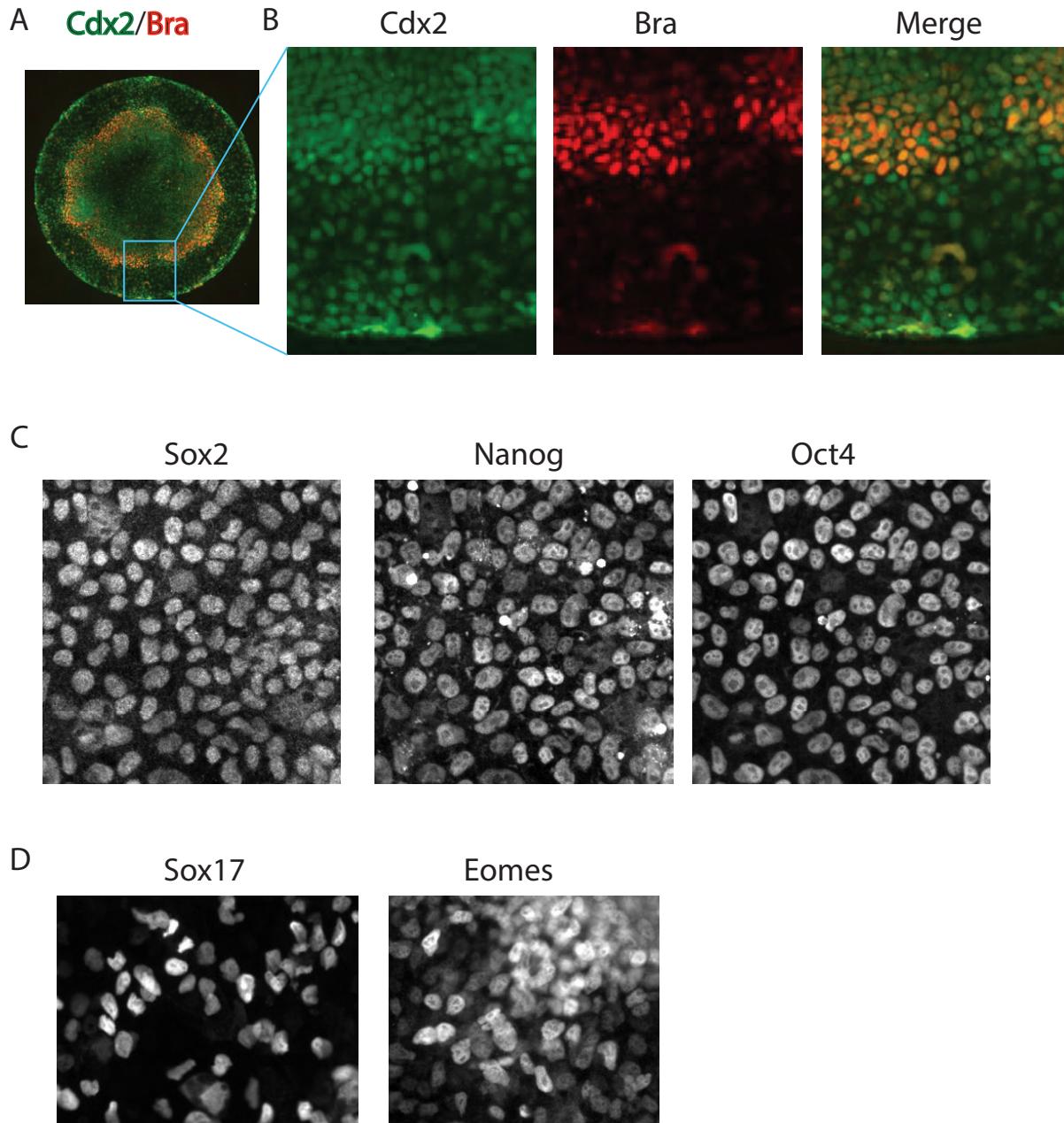
Patterns of differentiation are similar in other hESC lines and for other growth conditions. (A) Differentiation patterns of H1 cells. (B) Differentiation patterns of RUES1 cells. (C) Differentiation patterns of RUES2 cells grown on laminin 521 (D) Differentiation patterns of RUES2 cells grown in mTeSR1.

Supplementary Figure 10.



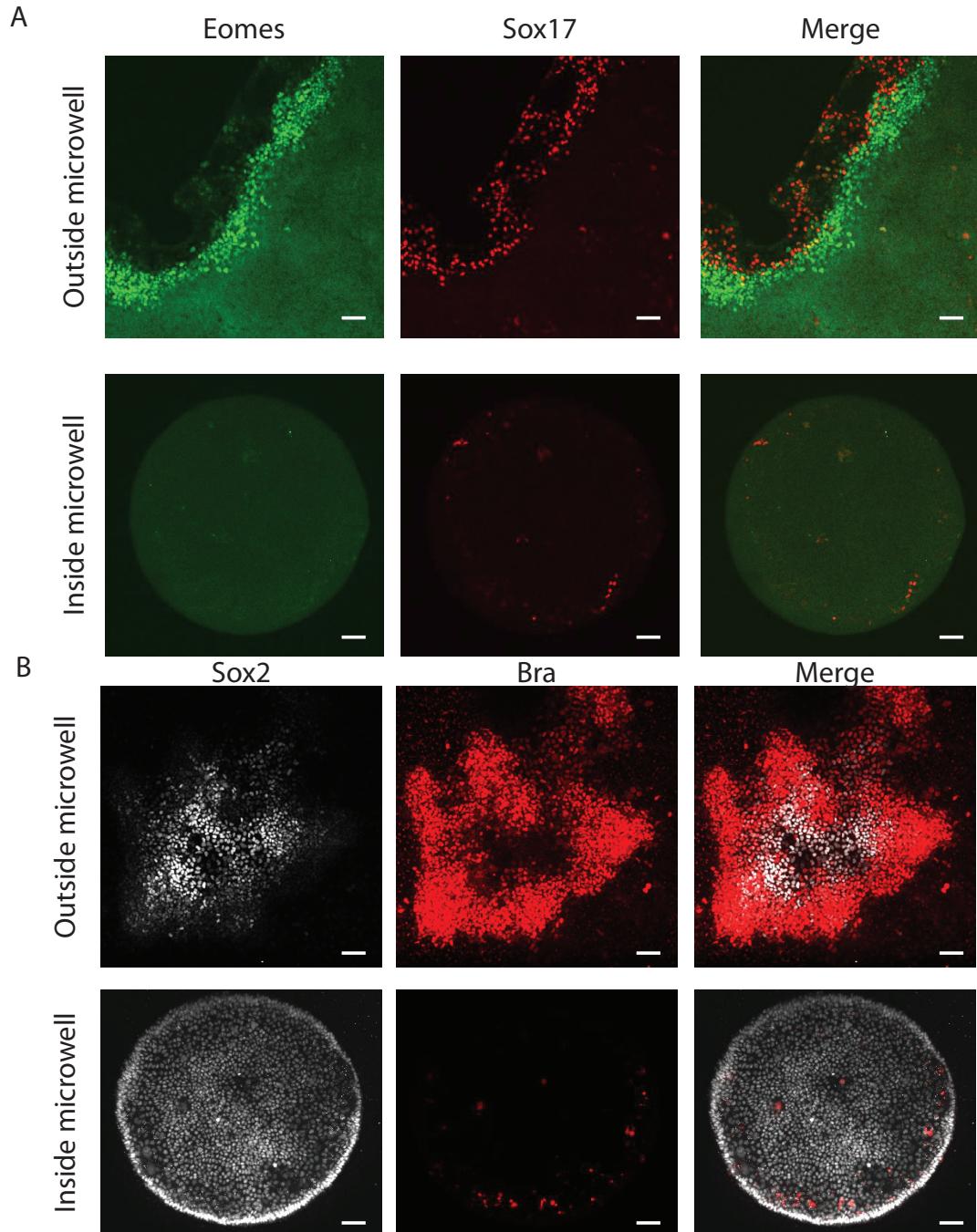
The dynamics of pattern do not vary with colony size. The indicated markers were quantified at the indicated times in both 1000 μm colonies (blue lines) and 500 μm colonies (red lines). Distances are indicated from the colony center, however, the lines for the 500 μm colonies were shifted by 250 μm to show their overlap with the 1000 μm colonies. That is, for the 500 μm colonies, a position of 250 μm is at the colony center.

Supplementary Figure 11.



The Cdx2 expressing cells can be subdivided by Brachyury expression. (A)
Immunofluorescence of a colony treated with BMP4 for 42 hours. **(B)** Higher magnification images of the region indicated in (A). Expression of Cdx2 extends from the colony border to encompass the region of mesoderm. **(C-D)** Higher magnification images of pluripotency factors (C) and additional differentiation markers (D) confirming nuclear localized staining.

Supplementary Figure 12.



Differentiation patterns require loss of a diffusible inhibitor across the colony boundary. To prevent diffusion of secreted proteins across the colony boundaries, cells were grown inside PDMS microwells approximately 1mm deep. As a control, cells were seeded in the same culture dish outside the microwells. Cells were treated with 50ng/ml BMP4 for 42 hours. (A) Mesendoderm differentiation is prevented inside the microwells where nearly all cells are negative for Eomes and Sox17. (B) Cells inside the microwells express elevated Sox2, the same fate found at the center of micropatterned disks of the same size.

Supplementary Table 1.

Antigen	Antibody	Dilution
pSmad1	Cell signaling 9516	1:100
Smad2	BD Bioscience 610842	1:100
Cdx2	Abcam Ab15258	1:50
Sox17	R & D Systems AF1924	1:200
Eomes	Abcam Ab23345	1:200
Brachyury	R & D systems AF2085	1:300
Sox2	Cell Signaling 3579	1:200
Oct4	BD Biosciences 611203	1:400
Nanog	Santa Cruz sc33759	1:100
Nanog	R & D Systems AF1997	1:200
Active β -catenin	Upstate 05-665	1:100
Gata6	Santa Cruz sc9055	1:200
pERK	Cell Signaling 9101	1:200
Snail	R & D Systems SC026	1:10
E-Cadherin	Cell Signaling 3195	1:200
EpCam	R & D Systems AF960	1:100

Primary antibodies and dilutions used for immunofluorescence in this study.

Reference

1. Warmflash, A. *et al.* Dynamics of TGF- β signaling reveal adaptive and pulsatile behaviors reflected in the nuclear localization of transcription factor Smad4. *Proc Natl Acad Sci USA* **109**, E1947–56 (2012).